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FLUORESCENT ANTIBODY TECHNIQUES APPLIED TO THE IDENTIFICATION OF DENGUE VIRUS IN INFECTED TISSUE

S. H. WATERMAN, T. P. MONATH

Department of Health and Human Services, Vector-Borne Viral Diseases Division, POB 2087, Fort Collins, 80522 Colorado, U.S.A.

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Summary. — We evaluated an indirect fluorescent antibody (FA) method for the detection of dengue virus antigen in infected mouse tissues. The biotin-avidin system [unlabeled antiviral antibody, biotinyl-anti-IgG and fluorescein conjugated avidin D(biotin-avidin system)] theoretically enhances the sensitivity of the FA method by amplifying the number of fluorescein particles attached indirectly to antigen. Using antibody endpoint titers in dengue-infected suckling mouse brain as an assay for sensitivity, we compared this three-step technique with the standard direct and two-step indirect FA techniques. Comparative tests were done on frozen sections of mouse brains with infectivity titers between 4.5 and 8.3 $\log_{10}$ LLC-MK2 cell PFU/g. Antibody endpoint titers with the biotin-avidin system were 2- to 8-fold higher than those obtained with the indirect and direct fluorescent antibody systems. The biotin-avidin system may be useful for rapid postmortem diagnosis of some fatal dengue hemorrhagic fever-dengue shock syndrome cases and perhaps also for early diagnosis of dengue by examination of leukocytes or biopsy material.

Key words: dengue virus; immunofluorescence; biotin-avidin system; diagnosis

Introduction

A severe form of the arboviral disease dengue fever, known as dengue hemorrhagic fever-dengue shock syndrome (DHF—DSS), is a major cause of morbidity among children in South-east Asia (Halstead, 1981); appearance of this disease in Cuba in 1981 (Centers for Disease Control, 1981) now poses a threat to the Caribbean region. Surveillance and epidemiologic studies to elucidate the pathogenesis of DHF—DSS and associated risk factors (e.g., prior heterologous infection, nutritional status, and infecting strain of dengue virus) depend upon accurate diagnosis of DHF—DSS cases (Halstead, 1980).

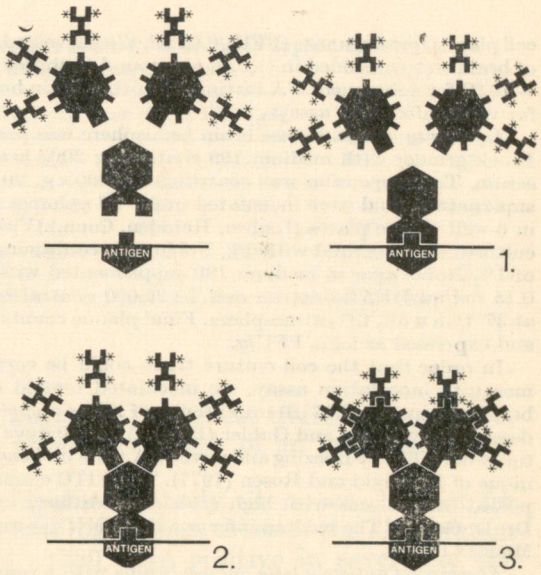

Fig. 1.
Schematic principle of the biotin-avidin fluorescent antibody test for detection of viral antigen
Step 1.: Attachment of primary antibody (hyperimmune mouse ascitic fluid) to antigen. Step 2.: Attachment to primary antibody of anti-mouse IgG covalently linked to biotin. Step 3.: Coupling of fluorescein-avidin D complex to biotin moieties on biotinylated anti-IgG

Reliable diagnosis of fatal DHF—DSS has often been difficult because death prevents the collection of convalescent-phase serologic specimens. The mosquito inoculation technique of Rosen and Gubler has considerably improved the ability to accurately diagnose fatal and nonfatal DHF—DSS cases by virus isolation (Rosen and Gubler, 1974). The drawback of this technique is that appropriate specimens may not be available and 2 weeks may be required to complete testing.

Fluorescent antibody (FA) tests, which require only a few hours to complete, have been used to detect dengue antigen in tissues of fatal DHF—DSS cases. However, attempts to use this diagnostic method on tissue from autopsy specimens have been mostly unsuccessful (Bhamarapravati and Boonyapucknavik, 1966). FA staining has been used effectively in the study of dengue pathogenesis in laboratory mice (Boonpucknavig *et al.*, 1981).

To define better the sensitivity of FA techniques, we compared a new FA system with the standard direct and indirect techniques for detecting viral antigen in frozen tissue sections of dengue-infected suckling mouse brain. The biotin-avidin (BA) system takes advantage of the high-binding affinity and amplification steps of biotin and avidin in a three-layer test (Bayer and Wilchek, 1978). The system uses 1) antiviral antibody, 2) biotin linked to anti-IgG, and 3) avidin labeled with fluorescein isothiocyanate (FITC) (Fig. 1). Biotin-avadin binding considerably increases the number of fluorescein particles attached to viral antigen.

Materials and Methods

Animals. Ten litters of 1 day-old Swiss suckling mice (NIH general purpose stain) were inoculated with dengue-2 virus New Guinea C strain (at the 24th mouse passage level) by the intracerebral (i.c.) route. The inoculum consisted of 1000 suckling mouse i.c. LD_{50} or LLC-MK2

cell plaque forming units (PFU)/0.02 ml. We harvested brains at 24-hour intervals; one hemisphere of brain was embedded in O.C.T. compound (Lab Tek Products, Naperville, Illinois*) and frozen —70 °C for subsequent FA testing; the other brain hemisphere was used to prepare a suspension for virus infectivity assays.

Infectivity assays. Mouse brain hemisphere was prepared as a 10% (w/v) suspension in a Ten Broek grinder with medium 199 containing 20% heat-inactivated (56 °C, 30 min) fetal bovine serum. The suspension was centrifuged (2000×g, 20 min 4 °C). Serial tenfold dilutions of the supernatant fluid were inoculated in 0.1 ml volumes into monolayer cultures of LLC-MK2 cells in 6-well plastic plates (Linbro, Hamden, Conn.). Virus was adsorbed for 1 hr at 37 °C, and the cultures were overlaid with 1% Noble agar containing 1% fetal bovine serum. A second overlay of 1% Noble agar in medium 199 supplemented with 1% fetal bovine serum, 2.0 g/l $NaHCO_3$, 0.15 mg/ml DEAE-dextran and 1 : 25,000 neutral red was added after three days incubation at 37 °C in a 5% CO_2 atmosphere. Final plaque counts were made and infectivity titers calculated and expressed as $\log_{10}$ PFU/g.

In order that the cell culture titers could be correlated with the putatively more sensitive mosquito inoculation assay, we inoculated tenfold dilutions of 10% infected suckling mouse brain suspension (0.17 µl) into groups of *Aedes aegypti* mosquitoes by the intrathoracic route, as described by Rosen and Gubler (1974). After 10 days incubation at 30 °C, the surviving mosquitoes were killed by freezing and direct FA tests on head-squashes were done according to the technique of Kuberski and Rosen (1977). The FITC conjugate for these FA tests was prepared from pooled human sera with high cross-reactive flavivirus antibodies and was kindly supplied by Dr. D. Gubler. The median infective dose (MID_{50}) was calculated using the method of Reed and Muench (1938-.

Fluorescent antibody tests. Mouse brains with a range of infectivity titers were used to prepare 6— to 8— micron frozen sections. The sections were placed on gelatin-coated glass slides and fixed in acetone (15 min, —20 °C). For the direct fluorescent antibody (DFA) rest we used conjugate prepared from a single human serum with a high titer of cross-reactive flavivirus antibodies kindly supplied by Dr. A. Chappell. As first antibody in the indirect fluorescent antibody (IFA) and the BA test systems, hyperimmune mouse ascitic fluid (MAF) against dengue-2 virus (New Guinea C) prepared by the Arbovirus Reference Branch, CDC, was used after twofold adsorption to 20% suspension of normal suckling mouse brain (NMB). The second antibody in the IFA test was FITC-conjugated goat antimouse IgG (Antibodies Inc., Davis, CA*). Second and third antibodies for the BA system were biotin-goat antimouse IgG (adsorbed with NMB) and FITC-conjugated avidin D, respectively (Vector Labs, Burlingame, CA*). The optimal dilutions of second and third reagents were determined by prior tests using St. Louis encephalitis virus in cell culture and infected mouse brains (unpublished data). Serial twofold dilutions of first antibody were added to the frozen sections to define the relative sensitivity of the three systems. Controls for the DFA tests were normal mouse brains stained with conjugate. We used normal MAF as first antibody on infected mouse brains as controls with the IFA and BA tests.

After each reagent was added, the slides were incubated for 30 min at 37 °C and then washed in phosphate-buffered saline pH 7.6 for 20 min. After the last stainig and washing, sections were covered with polyvinyl alcohol:glycerol under glass coverslips, and slides were examined with an Olympus BH2 microscope equipped with epifiuorescent blue light exciter filters IF-490 and EY 455 and barrier filter 0—530.

Statistical methods. We analyzed the data with one and two-day analysis of variance, analysis of covariance, and two-sample T statistical tests.

Results

Table 1 shows the results of the endpoint titrations of antibody in mouse brain for the three FA sastems. Comparison of the IFA and BA systems with the DFA tests is not strictly appropriate, because we used human antibody in the direct system, whereas we used a hyperimmune MAF for both the IFA and BA tests.

The infectivity titers of the mouse brains tested ranged from 4.5 to 8.3 $\log_{10}$ PFU/g. We were able to detect dengue viral antigen in all brains tested with

Table 1. Titer of antibody by three fluorescent antibody techniques applied to detection of dengue-2 viral antigen in mouse brain

FA method	Viral infectivity titer (log_{10} PFU/g*)							
	4.5	4.85	5.5	6.4	7.0	7.6	7.95	8.3
DFA	10	320	320	160–320	320	320	320	160–320
IFA	160	160	320	160	320	320	320	160
BA	1280	1280	640	640	640	1280	1280	1280

DFA = direct fluorescent antibody test; IFA = indirect fluorescent antibody test; BA = = biotin-avidin fluorescent antibody test.
* In LLC-MK2 cell monolayers.

all three FA systems except in one instance, the lowest titered mouse brain with the direct system. Fig. 2 is a photomicrograph of an infected neuron stained with the BA system.

The sensitivity, determined by endpoint antibody titers, was highest for the BA system; titers were 2— to 8 fold higherthan those of the IFA system ($p < 0.01$). Antibody dilutions of 1 : 1280 were positive in several of the brains tested with the BA system. Keeping in mind the different sources of antibody, the DFA and IFA tests demonstrated roughly the same endpoints (no statistically significant difference). The lowest titered mouse brain (4.5 PFU/g) was negative by the DFA system, positive by the IFA system, and interestingly, was positive at a 1 : 1280 dilution of antibody with the BA system despite the low infectivity titer. At optimal dilutions, the intensity of fluorescence was greater with the BA than with the DFA or IFA system.

In general, antibody titers did not vary greatly within FA test systems with change in tissue infectivity titer. We did not detect a great difference in proportions of infected cells between mouse brains. All brains tested in all three FA systems showed clusters of fluorescent cells except, again, the brain containing 4.5 log PFU/g, in which only occasional cells were positive.

Comparative infectivity assays by mosquito inoculation and LLC—MK2 plaque assay showed that the mosquito inoculation technique was approximately 100 times more sensitive. The titer in mosquitoes was $10^{8.66}$ MID_{50}/ml and in LLC—MK2 cells, 10^7 PFU/g, respectively.

Discussion

The accurate and rapid diagnosis of fatal and non-fatal cases of DHF—DSS is important to epidemiologic studies of risk factors for DHF—DSS. To improve diagnosis in fatal cases and study the pathogenesis of dengue infection, investigators have tried FA techniques in human autopsy material, but until very recently dengue antigen has proved difficult to detect. In a study in Thailand in the 1960's tissue from only 1 out of 21 fatal cases of DHF—DSS was positive for dengue antigen by immunofluorescence (Bhamarapravati and Boonyapucknavik, 1966). In a more recent study, tissues

from 2 of 4 fatal cases of DHF—DSS in Thailand were positive for dengue viral antigen by direct immunofluorescense (N. Bhamarapravati, pers. comm., 1981). In one case, fluorescent cells were demonstrated in liver, mesenteric lymph node, and spleen. In the other positive case, antigen was present in liver, lymph nodes, spleen, skin, lung, kidney, and jejunum. Antigen was found in some of the same tissue (spleen, kidney, liver, and lymph nodes) in a study of dengue pathogenesis in mice using direct immunofluorescent staining (Boonpucknavig *et al.*, 1981).

The results of our study provide evidence for an increased sensitivity of the BA system over standard DFA and IFA techniques for detecting dengue antigen in infected mouse brain tissue. The reagents used do not allow direct comparison between the BA and DFA tests but the BA system appears to offer an advantage over the direct test. Direct comparison between theIFA and BA tests is valid in this study, and the antibody endpoint titrations for the BA system are significantly (2- to 8-fold) higher than IFA endpoints ($p < 0.01$).

FA endpoint antibody titers did not appear sensitive to changes in tissue infectivity titer in our study. Our data suggest that, beyond a threshold of detectable antigen, antibody can be diluted to a fixed endpoint with each FA system. One might except changes in intensity of fluorescence of proportion of infected cells with change in tissue infectivity titer, but we had difficulty assesing these variables in this study.

The BA test demonstrated dengue antigen at 1 : 1280 dilution of antibody in mouse brain with an infectivity titer of 4.5 log PFU/g. Lower tissue was not available to us, but comparison on the three FA systems in tissues with lower infectivity titers would be important. It is possible that the BA system can detect antigen at lower concentrations than the one and two-step DFA and IFA techniques.

We used LLC—MK2 plaque assays to assess viral content of the mouse brains, but we should point out that the mosquito inoculation assay probably gives a closer indication of the true sensitivity of FA tests. The titer by mosquito inoculation assay of one mouse brain tested was approximately 100-fold higher than the titer of the same brain tested by cell culture assay. These results are consistent with those of Rosen and Gubler (1974).

Frozen sections of tissue from DHF cases may not always be available. Recent work has shown that FA tests can be used to detect viral antigen in formalin-fixed tissues treated with trypsin (Huang *et al.*, 1976; Johnson *et al.*, 1980). It may be possible to combine the BA FA system with this technique to improve retrospective diagnosis of fatal DHF cases.

The BA system may also be applicable to rapid diagnosis of non-fatal cases of dengue viral disease. Pathogenesis studies have implicated B lymphocytea as the primary sites of replications in natural dengue infections (Nisalak *et al.*, 1970). Lymphocyte-rich tissues, spleen, liver, and thymus, were FA positive in the studies by A. Nisalak (pers. comm., 1981). Dengue antigen and complement has been found by the DFA technique on the surface of lymphocytes separated from plasma of DHF patients (Boonpucknavig *et al.*, 1976). Dengue

antigen and complement appeared two days before shock or subsidence of fever. If further studies confirm an increased sensitivity of the BA FA system, then lymphocytes should be examined by this technique for early diagnosis.

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